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Serum CD63–PLAP placenta Extracellular Vesicles for Early Detection of Early-Onset Preeclampsia - Validation by ExoCounter in a Tanzanian Cohort

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Abstract

Early-onset preeclampsia (EOPE) is a severe pregnancy complication associated with high maternal and perinatal morbidity. Effective first-trimester screening followed by aspirin prophylaxis reduces EOPE incidence, but current risk stratification models (e.g., FMF) require advanced ultrasound and laboratory resources, limiting feasibility in low- and middle-income countries. Extracellular vesicles (EVs) from the placenta represent a promising, minimally invasive biomarker. We conducted a retrospective case–control analysis of prospectively collected serum and plasma samples from 75 Tanzanian women, including normotensive pregnancies (NP, n_serum = 19, n_plasma = 5), EOPE (n_serum = 19, n_plasma = 5), and late-onset preeclampsia (LOPE, n_serum = 20, n_plasma = 5). Using ExoCounter, we quantified psEVs with CD63–PLAP, CD10–PLAP, and CD10–CD63 antibody pairs. Quality control was applied to ensure reproducibility. Group comparisons, correlation with gestational age, regression analyses, and ROC curves were performed. Serum CD63–PLAP counts were significantly elevated in EOPE compared with NP and LOPE, while plasma counts showed higher variability. Serum CD63–PLAP correlated negatively with gestational age in NP but not in EOPE. Stratification indicated that the discriminatory power of CD63–PLAP emerged after 100 gestational days. ROC analysis showed that CD63–PLAP distinguished EOPE from NP or non-EOPE with AUC ~ 0.74–0.75, achieving ~ 95% sensitivity at 385.55 sEV/μL, although specificity remained modest (~ 44%). The addition of CD10–CD63, maternal age, BMI, or gestational age did not substantially improve discrimination. These findings support serum CD63–PLAP measured by ExoCounter as a promising candidate biomarker for EOPE, showing moderate discrimination with high sensitivity but limited specificity in this Tanzanian cohort. Its low sample-volume requirement and operational simplicity support further evaluation, particularly in settings where conventional multiparametric screening is difficult to implement. Larger prospective studies with independent validation are required to confirm its discriminatory performance, establish clinically relevant thresholds, and assess its potential role in EOPE screening.

Background

Preeclampsia (PE) is a pregnancy-specific hypertensive disorder that typically manifests after 20 weeks of gestation and is characterized by new-onset hypertension accompanied by proteinuria or maternal end-organ dysfunction. It affects approximately 2%–8% of pregnancies worldwide and remains a leading cause of maternal and perinatal morbidity and mortality, particularly in low-resource settings[1, 2]. In severe cases, complications such as eclampsia, HELLP syndrome, preterm birth, and intrauterine growth restriction substantially increase risks for both mothers and infants. Despite advances in obstetric care, the only definitive cure remains delivery of the placenta, often necessitating preterm birth with consequent neonatal complications[3].

Low-dose aspirin prophylaxis has emerged as an effective preventive strategy. Several randomized controlled trials and meta-analyses have demonstrated that initiating aspirin before 16 weeks of gestation significantly reduces the incidence of preeclampsia, especially the early-onset form, and improves both maternal and neonatal outcomes[4–6]. Accordingly, international guidelines, including

those from NICE and FIGO, recommend the administration of low-dose aspirin (typically 75–150 mg daily) in women identified as being at high risk[7]. Therefore, early and accurate identification of high-risk women is essential to facilitate timely and effective prophylaxis.

Clinically, preeclampsia is recognized as a heterogeneous condition with two major subtypes: early-onset preeclampsia (EOPE), occurring before 34 weeks of gestation, and late-onset preeclampsia (LOPE), occurring at or beyond 34 weeks. EOPE is more strongly associated with abnormal placentation and defective trophoblastic invasion during early pregnancy, leading to impaired uteroplacental perfusion. In contrast, LOPE is thought to be driven predominantly by maternal predisposition such as cardiovascular or metabolic risk factors, with relatively preserved placentation[8, 9]. The distinction is clinically relevant because EOPE is often more severe, with higher rates of adverse outcomes, and early recognition is particularly critical.

To improve early risk stratification, the Fetal Medicine Foundation (FMF) has developed a multiparametric first-trimester screening model that integrates maternal demographic and clinical characteristics, mean arterial pressure (MAP), uterine artery Doppler velocimetry, and maternal serum biomarkers such as placental growth factor (PlGF) [3, 10, 11]. This model has demonstrated high predictive performance, with detection rates for early-onset preeclampsia (EOPE) reaching 75%–90% at a false positive rate of 10%–20%[12]. Moreover, implementation of this strategy in combination with prophylactic aspirin has been shown to significantly reduce the incidence of EOPE in prospective trials[4]. However, the application of this model requires access to advanced ultrasound equipment, specialized biochemical assays, and trained personnel, which makes its widespread adoption challenging in low- and middle-income countries, where the burden of preeclampsia is greatest.

To address this gap, our group developed and validated a novel semiautomated assay, the ExoCounter, which enables sensitive and reproducible quantification of placental small extracellular vesicles (psEV) from minimal volumes of maternal serum. In our previous study, we demonstrated that psEV characterized by CD10–PLAP and CD63–PLAP antibody pairs were significantly elevated in women who subsequently developed EOPE, already in the first trimester, whereas such differences were not observed in LOPE[13]. This suggested that ExoCounter-derived biomarkers could provide a relatively simple, minimally invasive method to identify women at risk of EOPE in early pregnancy, offering an alternative screening tool particularly suitable for low-resource settings.

Building on this, we conducted a new retrospective case-control study using biobanked samples from pregnant women in Tanzania, including serum and plasma from normal pregnancies (NP), EOPE, and LOPE cases. This study had two objectives: first, to validate the reliability of ExoCounter-derived biomarkers in a distinct population from sub-Saharan Africa, thereby assessing cross-continental and cross-ethnic applicability; and second, to investigate whether these biomarkers could effectively discriminate EOPE from non-EOPE (NP + LOPE) in early pregnancy. By doing so, we aimed to further establish the potential role of ExoCounter in enhancing EOPE screening, both as a standalone tool in

resource-limited settings and as an adjunct to existing multiparametric models in high-resource countries.

Methods

Study Design, Participants and Ethical Approval

This study was a retrospective case–control analysis of prospectively collected and biobanked maternal blood samples from the Alliance for Maternal and Newborn Health Improvement (AMANHI) cohort in Tanzania. Women who subsequently developed EOPE or LOPE, together with women with normotensive pregnancies (NP), were included. Maternal venous blood samples had been prospectively collected at the first clinic visit before the diagnosis of preeclampsia. Twenty serum and five plasma samples from each group were available for the present analysis. After exclusion of one serum sample from the NP group and one from the EOPE group because of insufficient volume, the final serum cohort comprised 19 NP, 19 EOPE, and 20 LOPE samples, while the plasma cohort comprised five samples per group. Laboratory analyses were performed with investigators blinded to clinical information. The AMANHI study received ethical approval from local and institutional ethics committees, including the Zanzibar Medical Research and Ethics Committee, Zanzibar, Tanzania, and the Johns Hopkins University Institutional Review Board. The study protocols were also approved by the World Health Organization (WHO) Ethics Review Committee. All approvals were renewed annually throughout the study period.

EOPE and LOPE diagnosis criteria

Preeclampsia (PE) cases were identified using a structured decision-tree approach based on blood pressure measurements, proteinuria, antihypertensive treatment records, and evidence of maternal organ dysfunction. Women were classified as having PE if they demonstrated hypertension during pregnancy, defined as systolic blood pressure ≥ 140 mmHg or diastolic blood pressure ≥ 90 mmHg on at least two occasions after 20 weeks of gestation, or severe hypertension defined as systolic blood pressure ≥ 160 mmHg or diastolic blood pressure ≥ 110 mmHg at one or more time points during pregnancy, delivery, or postpartum. Proteinuria was defined as a urine dipstick result ≥ 1+, either at the same visit as elevated blood pressure or at a separate visit after exclusion of urinary tract infection. In women with severe hypertension but without concurrent proteinuria, cases were also classified as PE if there was evidence of maternal organ dysfunction after 20 weeks of gestation. Women receiving treatment for severe PE during delivery (e.g., magnesium sulfate or hydralazine) in the absence of complete blood pressure records were also considered PE cases following adjudication. PE cases were subsequently categorized as early-onset preeclampsia (EOPE) when diagnosis or delivery occurred before 34 weeks of gestation, and late-onset preeclampsia (LOPE) when occurring at or beyond 34 weeks of gestation. Supplementary Fig. 1 shows a Flow Chart used for diagnosis.

Maternal venous blood samples were prospectively collected at the first clinic visit prior to diagnosis and processed and stored in Tanzania as described below.

Plasma Sample Preparation

Maternal venous blood (7.5 mL) was collected into EDTA (purple-top) tubes by trained personnel and gently inverted to ensure proper anticoagulant mixing. Samples were processed within 30 minutes of collection and centrifuged at 2000 × g for 15 minutes at 4°C. Following centrifugation, plasma was carefully separated without disturbing the buffy coat layer and aliquoted into pre-barcoded 1.5 mL microtubes in 500 µL volumes (up to six aliquots per sample). All aliquots were immediately stored at −80°C until further analysis.

Serum Sample Collection

Maternal venous blood (7.5 mL) was collected into serum (brown-top) tubes and allowed to clot at room temperature for 30 minutes prior to processing. Samples were centrifuged at 2000 × g for 15 minutes at 4°C. The resulting serum layer was carefully aspirated, avoiding any cellular components, and aliquoted into pre-barcoded 1.5 mL microtubes in 500 µL volumes (up to six aliquots per sample). All aliquots were securely capped and stored at − 80°C until downstream analysis.

Sample coding

Twenty serum and five plasma samples from NP, EOPE, and LOPE were shipped to Oxford under temperature-controlled conditions. Samples were coded by the Tanzania team prior to shipment, and the assay operator at Oxford was blinded to all clinical information. Clinical data were shared with the Oxford team only after completion of sample analysis, prior to data analysis.

Antibody Biotinylation

PLAP mouse monoclonal antibody (NDOG2) was first buffer-exchanged using Amicon 10KDa MWCO centrifugal filters (Sigma-Aldrich, UFC201024) to remove preservatives such as sodium azide present in the storage buffer. The purified antibody was then conjugated with NH_2-reactive biotin (Dojindo, LK01) according to the manufacturer's instructions. Briefly, the antibody was incubated with freshly dissolved NH_2-biotin in reaction buffer at 37°C for 10 min, followed by repeated washes with WS buffer to eliminate unbound biotin. The resulting biotinylated antibody was recovered in WS buffer and stored at 4°C at a final concentration of approximately 1 mg/mL until use. The efficiency of biotinylation was subsequently verified using the Pierce™ Biotin Quantitation Kit (Thermo Fisher Scientific, 28005).

Beads Antibody Conjugation

Biotinylated PLAP and CD63 antibodies (Cosmo Bio Ltd, CAC-SHI-EXO-M02-B) were conjugated to avidin-coated nano-beads (Model No. BX-EAK3JA, JKCKENWOOD) following the manufacturer's instructions. Briefly, antibodies were diluted to 0.1 mg/mL in PBS, and the avidin beads were pre-washed three times with PBS to remove the storage buffer. The diluted antibody solution was then incubated with the washed beads under continuous gentle mixing at room temperature to allow biotin–avidin coupling. After binding, excess antibody was removed by repeated centrifugation and washing with storage buffer (HEPES-based). The final antibody–bead conjugates were resuspended in storage buffer and stored at 2–8°C until use.

Exocounter

Exocounter assays (JVCKENWOOD corp.) were performed essentially as described previously, with modifications to accommodate plasma and serum samples[13]. In brief, discs were first coated with 90 µL of CD63 (Biolegend, 353039) or CD10 (Miltenyi Biotec, 130-108-025) antibody (5 µg/mL) for 1 h at 37°C, followed by blocking with 200 µL of 0.1% casein in PBS with Tween 20 (PBST) for 1 h at room temperature to suppress nonspecific binding. Plasma or serum samples were thawed on ice, clarified by centrifugation (13,000g, 1 min), and diluted fourfold in PBST, after which 50 µL was applied to each well and incubated for 2 h at 37°C with gentle shaking. Bead-conjugated detection antibodies (20 µg/mL), prepared by sonicating and diluting the antibody–bead complexes in 0.1% casein/PBST, were then added at 50 µL per well and incubated for 90 min under the same conditions. Between each step, automated washing with PBST was carried out to remove unbound reagents, and final rinsing with deionized water was performed to reduce salt crystallization. After drying at 37°C, signals were recorded by the Exocounter optical system using a Blu-ray laser, and individual beads were quantified as discrete pulses by the detection circuit. Assay operators were blinded to all patient clinical information.

Statistical Analysis

Statistical analyses were performed using GraphPad Prism version 9 (GraphPad Software) and R version 4.3.1. For comparisons among three groups, normally distributed data were analyzed using one-way ANOVA with appropriate post hoc tests, whereas non-normally distributed data were assessed by the Kruskal–Wallis test. For two-group comparisons, normally distributed data were analyzed using the Student's *t*-test, and non-normally distributed data by the Mann–Whitney *U* test. Linear regression analysis was applied to evaluate the association between Exocounter counts and gestational age. Receiver operating characteristic (ROC) curves and the corresponding area under the curve (AUC) values were calculated using the R package pROC[14], and optimal cut-off thresholds were determined according to the Youden Index. The 95% confidence intervals for AUC values were calculated using the DeLong method, whereas confidence intervals for sensitivity and specificity at the Youden Index-derived cut-off were calculated using the exact binomial method. For multivariable modeling, least absolute shrinkage and selection operator (LASSO) regression was employed to identify informative predictors. A p-value < 0.05 was considered statistically significant. Given the exploratory nature of the study, the 100-

day gestational-age stratification and ROC-derived cut-off were identified and evaluated within the same dataset and were not independently validated.

Result

Baseline characteristics and quality control of samples

A total of 19 serum and 5 plasma samples were included for both the NP and EOPE groups after exclusion of one serum sample in each group due to insufficient volume. The LOPE group comprised 20 serum and 5 plasma samples. Clinical characteristics of the three groups are summarized in Table 1. Maternal age, body mass index, gestational age at delivery, and birthweight differed significantly among the three groups (all $p < 0.01$), whereas parity and gestational age at sampling did not differ significantly. All EOPE pregnancies resulted in preterm delivery, whereas this was rare in the LOPE group and absent in the NP group.

Table 1
Clinical characteristics of the study population. **p < 0.01, ****p < 0.0001.

Maternal age (years)	**NP**	**EOPE**	**LOPE**	**P**
	25.52 ± 0.50	**30.40 ± 1.60**	**29.68 ± 1.19**	**
Body mass (kg/m^2)	22.12 ± 0.41	26.80 ± 1.53	26.47 ± 0.97	**
Birth weight (g)	3294 ± 56.42	1918 ± 154.2	3217 ± 132.1	****
Parity	3.00 ± 0.24	4.35 ± 0.61	4.78 ± 0.68	0.233
Gestational age at sampling	104.6 ± 4.50	100.7 ± 5.13	115.8 ± 3.06	0.084
Gestational age at birth	276.4 ± 1.24	229.0 ± 3.94	272.0 ± 1.96	****
Rate of preterm bith	0%	100%	8%	****
Rate of FGR				

Quality control of Exocounter measurements was performed according to the flowchart shown in Fig. 1A. In the first round of testing, the majority of samples met the pre-specified QC criteria. A small number of samples failed the first round and were re-tested. After the second round of QC, all samples passed with acceptable coefficients of variation, ensuring robust data quality for downstream analysis (Supplementary Table 1).

Serum CD63–PLAP is elevated in EOPE and antibody pair counts show consistency

Exocounter analysis was next performed to compare psEV levels across the three groups using different antibody pairs (Fig. 1B–D). In serum samples (Fig. 1B), the abundance of CD63–PLAP–positive vesicles

was significantly elevated in women who subsequently developed EOPE compared with NP and LOPE, whereas no significant differences were observed for CD10–PLAP or CD10–CD63 pairs. By contrast, in plasma samples (Fig. 1C), CD63–PLAP counts did not differ significantly among groups. Notably, overall EV counts detected in plasma were consistently higher than in serum, approximately two- to three-fold, regardless of antibody pair. To examine whether the three antibody pairs reflected consistent patterns within individuals, we compared their counts across matched samples (Fig. 1D). A concordant trend was observed, whereby individuals with high counts for one antibody pair generally exhibited elevated counts for the others, suggesting internal consistency of the Exocounter measurements.

Both CD10–CD63 and CD63–PLAP EV counts are elevated in EOPE compared with non-EOPE

To further explore the discriminatory ability of EV markers for EOPE, we combined NP and LOPE groups into a non-EOPE reference group, given the distinct pathophysiology of EOPE and the fact that samples were collected in early pregnancy. As shown in Fig. 2, EOPE cases demonstrated significantly elevated counts of CD10–CD63 and CD63–PLAP positive EVs in serum compared with non-EOPE, whereas CD10–PLAP showed no significant difference. In plasma, no significant group differences were detected for any of the antibody pairs, although the absolute EV counts were generally 2–3 fold higher than those measured in serum. Given the limited number of plasma samples, these findings should not be interpreted as evidence of superior discriminatory performance of serum over plasma.

Stratified analyses show consistent elevation of CD63–PLAP in EOPE across maternal age and BMI subgroups To determine whether the observed differences in EV counts were attributable to disease status or maternal baseline characteristics, we performed stratified analyses according to maternal age and BMI (Fig. 3A–H). Among women younger than 25 years, EOPE showed significantly higher levels of CD10–CD63 and CD63–PLAP compared with NP, and higher CD10–PLAP compared with LOPE (Fig. 3A). When grouped as EOPE versus non-EOPE, all three antibody pairs were significantly elevated in EOPE within this age stratum (Fig. 3B). In women aged 26–29 years, no significant differences were observed across NP, EOPE, and LOPE, but EOPE displayed higher CD63–PLAP compared with non-EOPE (Fig. 3C–D). In the subgroup aged ≥ 30 years (no NP cases), EOPE exhibited significantly increased CD63–PLAP relative to LOPE (Fig. 3E). Stratification by BMI showed that in women with BMI ≤ 24.9, EOPE had significantly higher CD10–CD63 and CD63–PLAP compared with non-EOPE (Fig. 3F–G), while in women with BMI > 24.9 (without NP), EOPE again displayed elevated CD63–PLAP compared with LOPE (Fig. 3H). Taken together, these findings show that elevated CD63–PLAP levels in EOPE were observed across several maternal age and BMI strata, although these stratified analyses do not establish independence from these maternal characteristics.

CD63-PLAP correlates negatively with gestational age in NP

To investigate whether EV counts change with gestational age during early pregnancy, we performed correlation and linear regression analyses between gestational age at sampling, expressed in days, and the counts of each antibody pair within each clinical group. As shown in Fig. 4, a strong negative correlation was observed between gestational age and CD63–PLAP in the NP group ($r = -0.763$, $R^2 = 0.582$, $p = 0.0002$), indicating that CD63–PLAP levels decrease as pregnancy progresses in normal pregnancies. By contrast, a weak positive correlation was found between gestational age and CD10–CD63 counts in the LOPE group ($r = 0.432$, $R^2 = 0.186$, $p = 0.0287$). No significant associations were detected for other antibody pairs in EOPE or LOPE groups. These findings suggest that the effect of gestational age on EV profiles, relative to NP, varies by preeclampsia phenotype.

CD63–PLAP elevation in EOPE becomes significant after 100 days of gestation

Given that regression analysis suggested a negative correlation between CD63–PLAP counts and gestational age in the NP group but not in EOPE, we further stratified the samples by gestational age at blood collection (≤ 100 days vs. >100 days, Fig. 5A–D). In women sampled before 100 days, EOPE tended to have relatively higher CD63–PLAP levels; however, the differences were not statistically significant, either in comparisons among NP, EOPE, and LOPE (Fig. 5A), or between EOPE and non-EOPE (Fig. 5B). By contrast, among women sampled after 100 days of gestation, CD63–PLAP counts were higher in EOPE than in both NP and LOPE (Fig. 5C). In the EOPE-versus-non-EOPE comparison, both CD10–CD63 and CD63–PLAP counts were significantly higher in EOPE (Fig. 5D). These exploratory findings suggest that differences in CD63–PLAP were more apparent after 100 days of gestation.

Serum CD63–PLAP discriminates EOPE with high sensitivity but limited specificity Building on the observation that serum CD63–PLAP levels were elevated in EOPE, we next evaluated its discriminatory performance for early detection. ROC analysis demonstrated that CD63–PLAP alone distinguished NP from EOPE with an AUC of 0.74 (95% CI 0.58–0.90) (Fig. 5E). The Youden Index identified an optimal threshold of 385.55 sEV/μL, corresponding to a sensitivity of 0.95 (95% CI 0.74-1.00) and a specificity of 0.42 (95% CI 0.20–0.67) for distinguishing EOPE from NP. When comparing EOPE with non-EOPE, CD63–PLAP yielded a similar AUC of 0.75 (95% CI 0.61–0.88) (Fig. 5F), with sensitivity of 0.95 (95% CI 0.74-1.00) and specificity of 0.44 (95% CI 0.28–0.60) at the same threshold. We further explored multivariable models by combining CD63–PLAP with additional parameters, including CD10–CD63, gestational age, BMI, and maternal age. The resulting AUC values ranged from 0.72 to 0.80 (Supplementary Fig. 2), which did not substantially improve discrimination. These findings indicate that serum CD63–PLAP shows moderate discriminatory performance for EOPE in this cohort, with high sensitivity but limited specificity, while the addition of the evaluated variables did not substantially improve discrimination.

Discussion

In this study, we evaluated psEVs in maternal serum and plasma during early pregnancy among Tanzanian women using the ExoCounter platform. We demonstrated that CD63–PLAP counts were

significantly elevated in women who subsequently developed EOPE compared with NP and LOPE. ROC analyses yielded AUCs of approximately 0.74, with a threshold of 385.55 sEV/μL achieving 95% sensitivity and 44% specificity.

Baseline clinical characteristics differed among the NP, EOPE, and LOPE groups, particularly with respect to maternal age and BMI. This aligns with the established literature showing that advanced maternal age and overweight/obesity substantially increase the risk of preeclampsia[15–20]. Furthermore, the EOPE group had a 100% rate of preterm birth, consistent with reports that EOPE is associated with more severe clinical manifestations and worse perinatal outcomes compared to LOPE [2]. These findings underscore the urgent need for effective early screening tools that can identify high-risk women during the first trimester.

Our results corroborate our previous study in a UK cohort, which identified CD63–PLAP and CD10–PLAP psEVs as elevated in EOPE during 10–12 weeks of gestation[13]. The difference in pathophysiology between EOPE and LOPE highlights the importance of distinguishing these two entities. In contrast to EOPE, which arises from defective placentation and impaired spiral artery remodelling leading to placental hypoxia, reperfusion injury, and systemic endothelial dysfunction, LOPE is primarily associated with maternal constitutional or metabolic factors and develops later in pregnancy[8, 21–24]. In this context, biomarkers detectable in the first trimester are particularly relevant for EOPE but not for LOPE. By grouping NP and LOPE together as non-EOPE, we confirmed that CD63–PLAP was significantly elevated in EOPE, reinforcing the potential of ExoCounter to identify high-risk pregnancies early.

Compared with our earlier study, several important differences emerged. First, overall EV counts were lower in the present cohort, which may primarily reflect differences in sample processing, particularly centrifugation conditions. A higher g-force (2000 × g for 15 minutes) was used for the Tanzania serum samples, compared with 1300 × g for 5 minutes in the earlier study. In addition, the broader sampling window (8–20 weeks versus 10–12 weeks previously) may have contributed to the observed differences. Consistent with this, regression analyses showed that CD63–PLAP levels decreased with gestational age in NP, suggesting a time-dependent decline that may have influenced EV counts. Second, CD10–PLAP did not consistently differentiate EOPE in the current study, which may be related to diagnostic criteria for preeclampsia compared with the International Society for the Study of Hypertension in Pregnancy (ISSHP) definitions applied in our earlier work[25]. Furthermore, although plasma psEV counts were approximately two to three times higher than those in serum, no significant differences between clinical groups were detected in the small plasma subset. Given the limited number of plasma samples (n = 5 per group), direct conclusions regarding the relative discriminatory performance of serum and plasma cannot be drawn. In the previous study, plasma samples were prepared by centrifuging whole blood at 1500 × g for 10 minutes, followed by a second centrifugation at 20,000 × g for 2 minutes at room temperature, to minimize platelet activation and the release of platelet-derived EVs. In contrast, Tanzania samples were processed using a single centrifugation step at 2000 × g for 15 minutes at 4°C. Platelet activation and the release of platelet-derived EVs under these conditions may have contributed to the greater variability observed in the plasma measurements[26]. Importantly,

stratified analyses by age and BMI showed that elevated CD63–PLAP levels were observed across several subgroups, supporting the consistency of the association across different maternal demographic strata.

Gestational age exerted important effects on psEV dynamics. In NP, CD63–PLAP correlated negatively with gestational age, while this relationship was absent in EOPE and LOPE. These findings suggest that physiological psEV release may normally decline as pregnancy progresses; however, in EOPE, persistent placental dysfunction may drive sustained and aberrant EV release into the maternal circulation. These findings align with the two-stage model of EOPE pathogenesis, in which placental stress triggers systemic maternal responses via extracellular vesicle signaling[27–29]. The different pattern in LOPE may reflect its distinct, later-onset pathophysiology[30, 31]. Our gestational-age stratification suggested that differences in CD63–PLAP were more apparent after 100 days (~ 14 weeks); however, this exploratory observation requires independent validation before an optimal screening window can be defined. At the Youden Index-derived threshold of 385.55 sEV/μL, sensitivity approached 95%, although specificity remained limited. From a clinical perspective, high sensitivity is particularly relevant for an early screening biomarker, as failure to identify women at high risk may result in missed opportunities for prophylaxis with low-dose aspirin (75–150 mg daily) when initiated before 16 weeks[32, 33]. Given that the discriminatory difference in CD63–PLAP became more apparent after approximately 100 gestational days (~ 14 weeks), ExoCounter-based assessment during this window could potentially support timely identification of women who may benefit from further risk assessment and preventive management. Meta-analyses and large randomized controlled trials, including the ASPRE trial, have demonstrated that aspirin reduces the incidence of EOPE when initiated early in high-risk pregnancies[11, 34]. Nevertheless, the limited specificity and lack of independent validation indicate that the current threshold should be considered exploratory and requires prospective validation before clinical implementation.

These findings are particularly relevant for low- and middle-income countries (LMICs), where preeclampsia contributes disproportionately to maternal morbidity and mortality[35, 36]. The Fetal Medicine Foundation screening model, which combines maternal characteristics, uterine artery Doppler, and serum biomarkers such as PlGF, has achieved detection rates of 75–90% in high-income countries[12, 37, 38]. However, FMF screening requires advanced ultrasound equipment and specialized laboratory assays, limiting its implementation in resource-poor settings[39–41]. By contrast, ExoCounter requires only microliter volumes of serum, minimal processing, and yields reproducible results, supporting its potential feasibility in LMICs. In high-resource settings, CD63–PLAP could be evaluated as an adjunct to existing FMF screening models.

Strengths of this study include the use of prospectively collected pre-diagnostic samples, blinded laboratory analysis, application of a novel EV-based biomarker platform, and evaluation in an African population. Limitations include modest sample sizes, particularly in subgroup analyses, the broader gestational age sampling window, and less standardized diagnostic criteria compared to prior work. In addition, the 100-day gestational-age threshold and the 385.55 sEV/μL cut-off were derived and evaluated within the same dataset and were not independently validated; these findings should therefore

be considered exploratory. The absence of complete newborn sex data limited accurate classification of fetal growth restriction (FGR) versus preterm birth, thereby precluding subgroup analysis. Larger multicenter studies are needed to validate gestational-age–specific thresholds, further evaluate discriminatory performance, and test real-world implementation of ExoCounter in both LMICs and high-income contexts.

In conclusion, serum CD63–PLAP measured by ExoCounter during early pregnancy showed moderate discriminatory performance for EOPE in this Tanzanian cohort, with high sensitivity but limited specificity. Its minimal sample requirement and operational simplicity support further evaluation as a potential component of EOPE screening. Larger prospective studies with independent validation are required to confirm its discriminatory performance, establish clinically relevant thresholds, and assess its utility in real-world settings.

Declarations

Ethics approval and consent to participate

The AMANHI study received ethical approval from local and institutional ethics committees, including the Zanzibar Medical Research and Ethics Committee, Zanzibar, Tanzania, and the Johns Hopkins University Institutional Review Board. The study protocols were also approved by the World Health Organization (WHO) Ethics Review Committee. All approvals were renewed annually throughout the study period. Having established MTA between University of Oxford and Professor SS, the allocated AMANHI samples were adopted under the Central Oxfordshire Research Ethics Committee C (REFS 07/H0607/74 & 07/H0606/148 & 14/SC/0106) in accordance with the Declaration of Helsinki principles. Written informed consent was obtained from all participants.

Consent for publication

Not applicable

Availability of data and materials

Not applicable

Competing interests

Authors declare that is not competing interests.

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Author Contributions

YJ and WZ conducted reagent preparation, assay validation, blinded sample analysis, data analysis, and manuscript preparation. SD, AD, BBK, MSA, and UD provided serum and plasma samples from the AMANHI study. SD critically reviewed the manuscript. AS provided placenta perfusion-derived small extracellular vesicles for initial reagent development and assay validation. WZ and SS provided resources and supervised the study.

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Figures

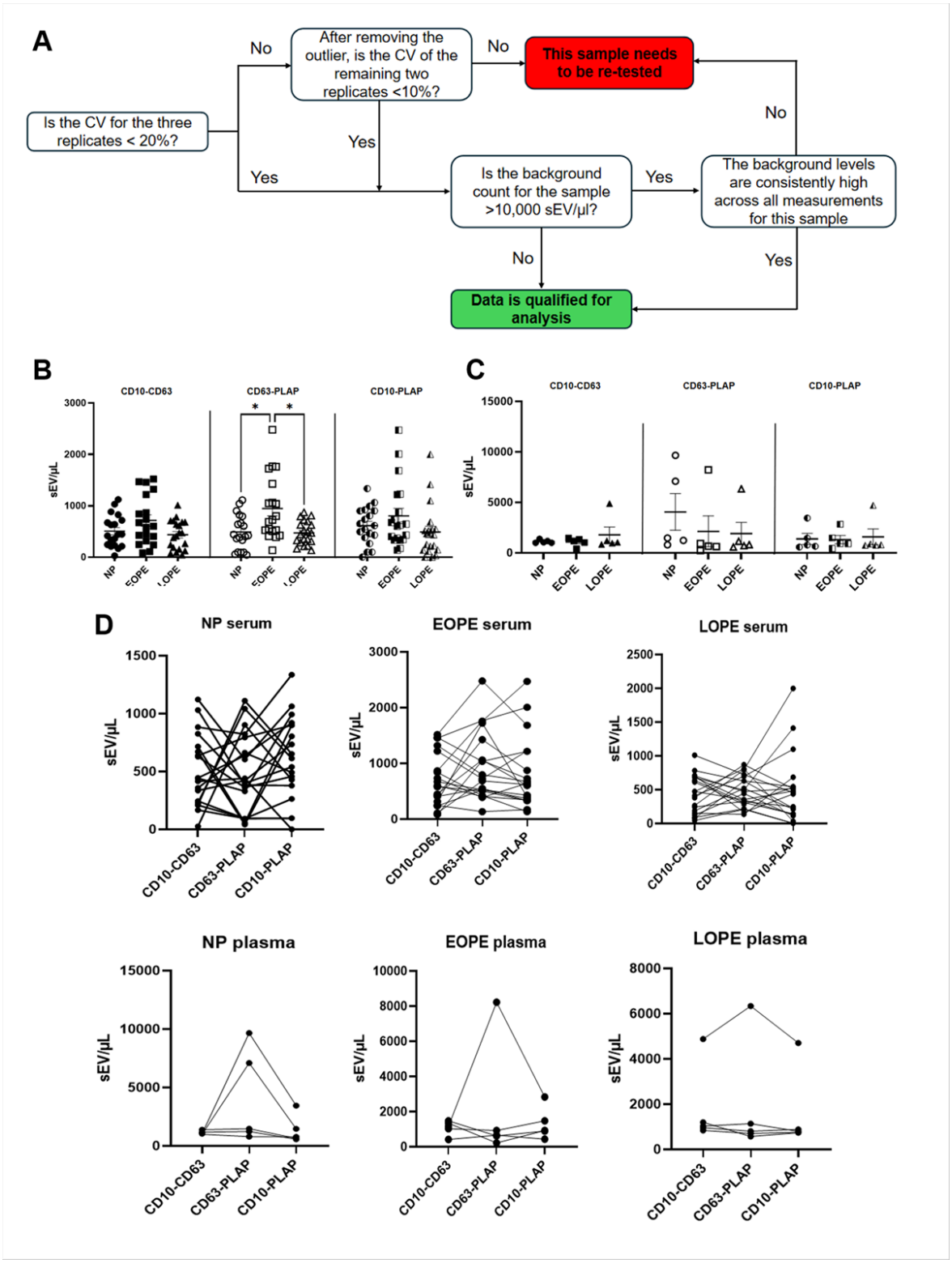


Figure 1

QC of Exocounter measurements and comparison of antibody pair counts among NP, EOPE, and LOPE.

(A) Flowchart of the quality control (QC) procedure. (B) Serum EV counts measured by Exocounter using CD10–PLAP, CD10–CD63, and CD63–PLAP antibody pairs. (C) Plasma EV counts measured by Exocounter using the same antibody pairs. (D) Distribution of EV counts across the three antibody pairs

in matched samples. NP, normotensive pregnancy; EOPE, early-onset preeclampsia; LOPE, late-onset preeclampsia.

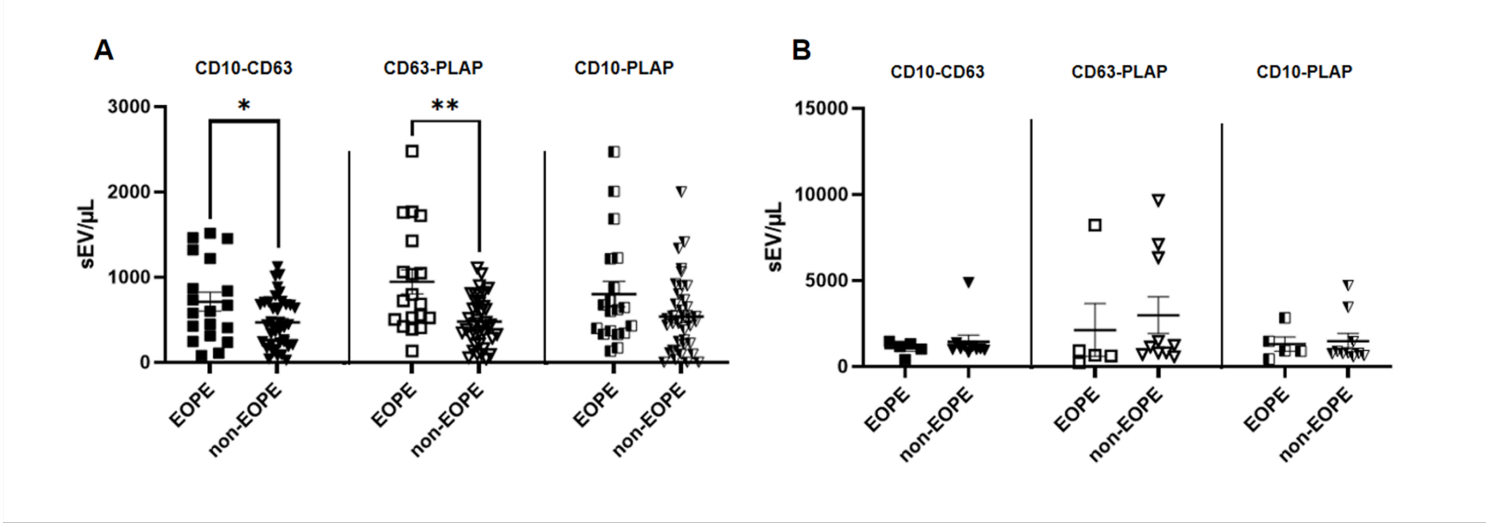


Figure 2

Comparison of Exocounter counts between EOPE and non-EOPE (NP + LOPE) groups.
(A) Serum samples. (B) Plasma samples.

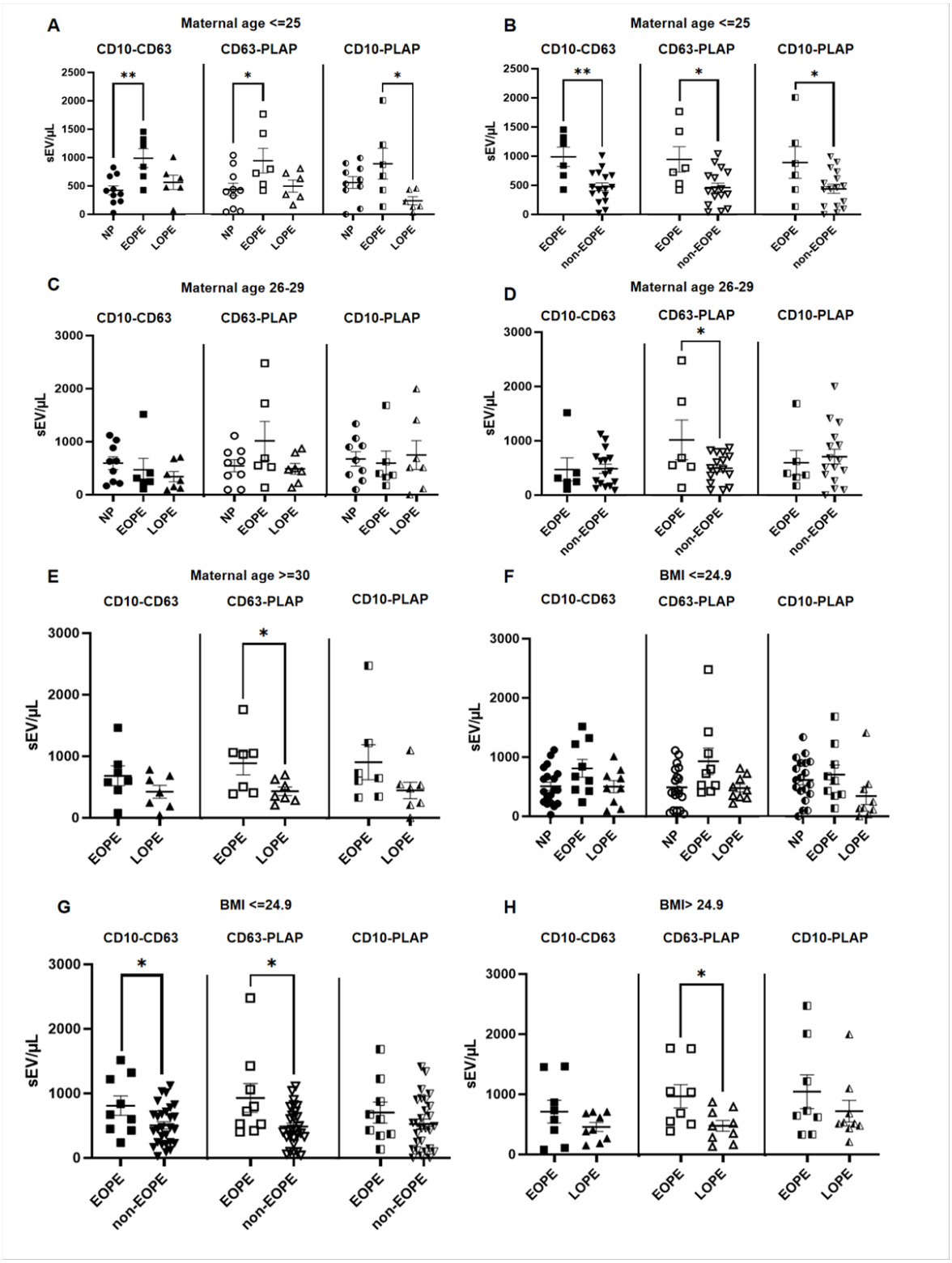


Figure 3

Stratified analyses of EV counts by maternal age and BMI.

(A) Comparison of NP, EOPE, and LOPE in women <25 years. (B) Comparison of EOPE versus non-EOPE in women <25 years. (C–D) Analyses in women aged 26–29 years. (E) Comparison of EOPE and LOPE in women aged ≥30 years. (F–G) Analyses in women with BMI ≤24.9. (H) Comparison of EOPE and LOPE in women with BMI >24.9. Each dot represents an individual sample.

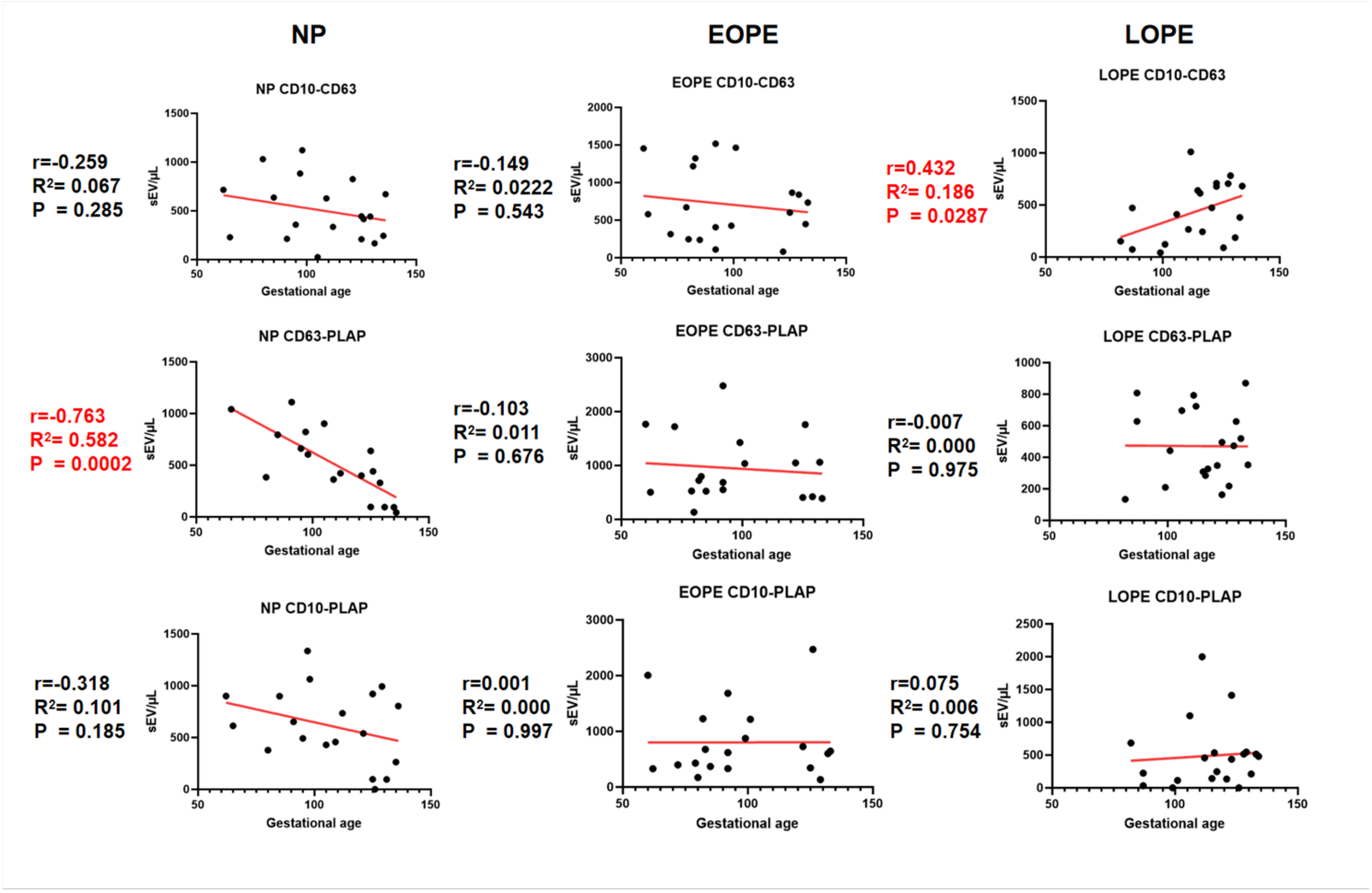


Figure 4

Correlation analysis between gestational age at sampling and EV counts across different antibody pairs.

Scatter plots showing the relationship between gestational age at sampling (days) and counts of EVs detected by CD10–CD63, CD63–PLAP, and CD10–PLAP antibody pairs in NP, EOPE, and LOPE groups. Each dot represents an individual sample. Red lines indicate linear regression fits, and corresponding r, R^2, and p-values are shown in the panels.

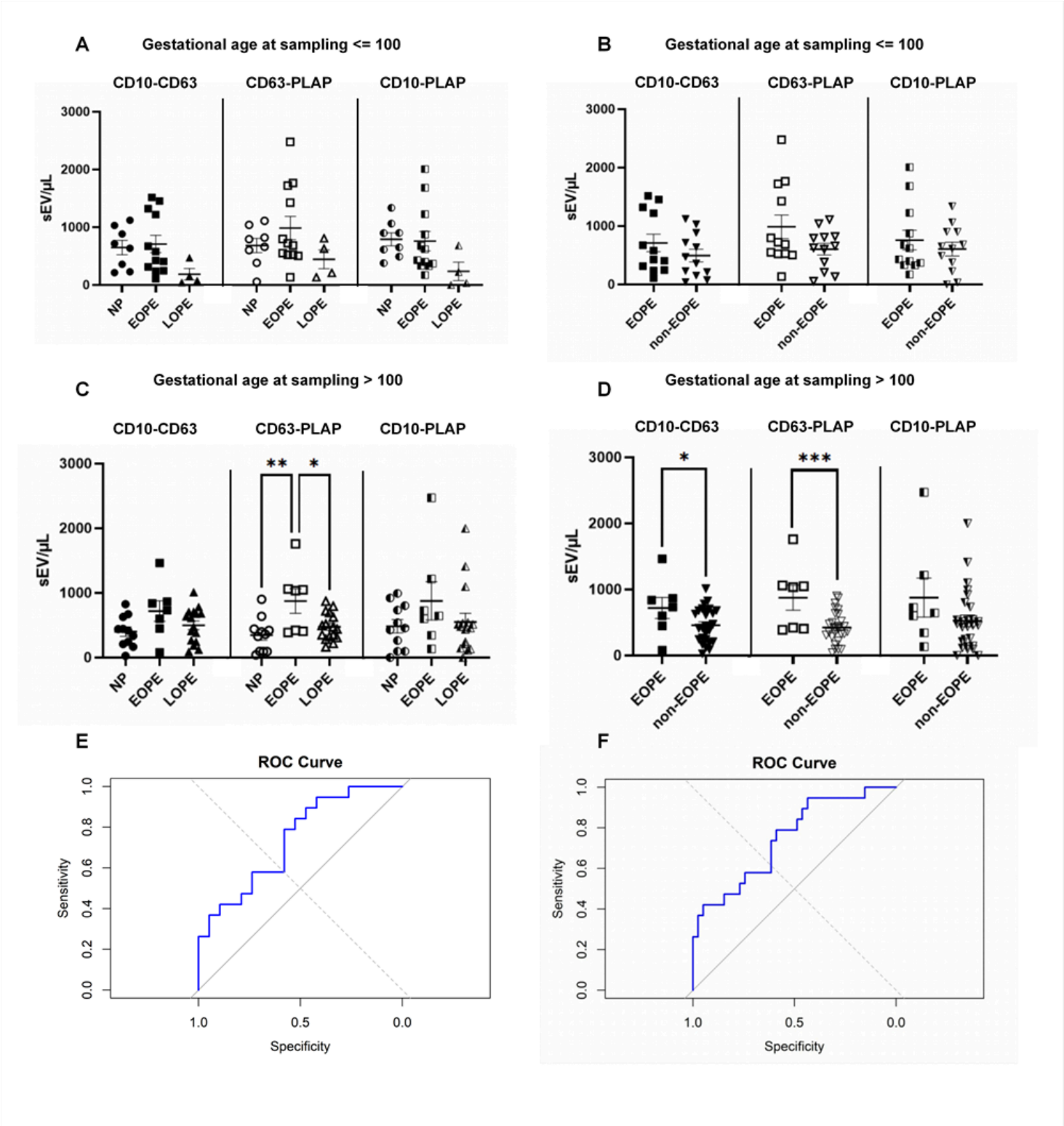


Figure 5

Comparison of EV counts stratified by gestational age at sampling.

(A–B) EV counts of CD10–CD63, CD63–PLAP, and CD10–PLAP in NP, EOPE, and LOPE groups (A), and in EOPE versus non-EOPE groups (B), for samples collected at ≤100 gestational days. (C–D) EV counts of CD10–CD63, CD63–PLAP, and CD10–PLAP in NP, EOPE, and LOPE groups (C), and in EOPE versus non-

EOPE groups (D), for samples collected at >100 gestational days. (E) ROC curve of serum CD63–PLAP-positive psEVs for discriminating EOPE from NP. (F) ROC curve of serum CD63–PLAP-positive psEVs for discriminating EOPE from non-EOPE pregnancies (NP + LOPE).

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- SupplementaryTable1.docx
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- SF2.tif